PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/68, C07H 19/04, 21/00 — A1

(11) International Publication Number: **WO 00/37674**

(43) International Publication Date: 29 June 2000 (29.06.00)

(21) International Application Number: PCT/GB99/04208

(22) International Filing Date: 20 December 1999 (20.12.99)

(30) Priority Data:
9827908.6 19 December 1998 (19.12.98) GB

(71) Applicant *(for all designated States except US)*: THE VICTORIA UNIVERSITY OF MANCHESTER [GB/GB]; Oxford Road, Manchester M13 9PL (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DOUGLAS, Kenneth, Thomas [GB/GB]; Higher Shore Cottage, Higher Shore Road, Littleborough OL15 9LW (GB). BICHENKOVA, Elena, Vladimirovna; [LV/GB]; 2 Egerton Court, Upper ParkRoad, Manchester, M14 5SL (GB).

(74) Agent: ATKINSON, Peter, Birch; Marks & Clerk, Sussex House, 83-85 Mosley Street, Manchester M2 3LG (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NUCLEIC ACID SEQUENCING METHOD

(57) Abstract

A method of determining the sequence of a nucleic acid involves the use of an excimer or exciplex as a label. The method may comprise effecting a single copying reaction of the nucleic acid to be sequenced using chain extending nucleotides and an appropriate number of chain terminating nucleotides each labelled with the partners of a detectably different excimer or exciplex, the method further comprising separating the labelled fragments produced and detecting the labels to determine the sequence of the nucleic acid.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

NUCLEIC ACID SEQUENCING METHOD

The present invention relates to a method of sequencing nucleic acids.

The analysis of DNA structure and expression has been greatly facilitated by the development of methods for the sequencing of nucleic acid molecules. Such sequencing methods generally involve generating labelled nucleic acid fragments derived from the nucleic acid being sequenced and detecting the fragments to determine the sequence of the nucleic acid. Examples of such methods for sequencing DNA are the "Maxam and Gilbert" chemical cleavage method and the "Sanger" dideoxy method.

The "Sanger" method involves using a polymerase to copy single stranded DNA to be sequenced. The incubation mixture comprises the four deoxy-ribonucleotide triphosphates required for DNA synthesis together with a 2', 3'-dideoxy analogue of one of the triphosphates (or other "stop" or chain terminating molecule). Either the analogue or the "normal" nucleotides may be radiolabelled. The incorporation of the stop molecule into the synthesised DNA strand blocks further growth of the strand because it lacks the 3'-hydroxyl terminus needed to form the next phosphodiester bond. Therefore, fragments of various lengths are produced in which a stop molecule is at the end of the 3' end of the DNA strand. Four such sets of chain-terminated fragments may then be subjected to size separation by electrophoresis and the base sequence of the newly synthesised DNA sequence read from an autoradiogram, indicating the sequence of the template DNA.

The use of a radiochemical label in the "Sanger" method (and also in the "Maxam and Gilbert" method) is a disadvantage and a variant of the "Sanger" method has been developed using fluorescent rather than radiochemical labels.

Fluorescence methods also have advantages based on speed of detection and the possibility of measuring more than one event simultaneously. In the case of fluorescent tagging, each of the common bases can be modified by a different

fluorophore in principle and used as a chain terminator. Alternatively a fluorescent label can be attached to the 5'-end of the primer. Fluorescently labelled oligonucleotides are detected by the characteristic colour of their emitted fluorescence.

One disadvantage of such an approach is the need for fluorophores whose emission bands do not significantly overlap, to minimise background and false identifications of base identity.

It is an object of the present invention to obviate or mitigate the abovementioned disadvantages.

According to the first aspect of the present invention there is provided a method of determining the sequence of a nucleic acid involving the use of label wherein the label is an excimer or exciplex.

According to a second aspect of the present invention there is provided a method of sequencing a nucleic acid by generating labelled nucleic acid fragments derived from the nucleic acid and detecting the fragments to determine the sequence of the nucleic acid wherein the label of the fragments is an excimer or an exciplex.

An excimer is a fluorescent complex formed when two identical complex forming partners (e.g. pyrene) are in the correct positional relationship relative to each other and photo-irradiated (e.g. by a laser). Excimers have a red-shifted emission maximum relative top the partners from which they are formed and are detectably different therefrom.

An exciplex is a heterocomplex analogue of an excimer and as such the exciplex forming partners are different from each other, one acting as a donor and the other as an acceptor. As for excimers, exciplexes also have a red shifted emission relative to the partners from which they are formed and are therefore detectably different therefrom.

Excimers and exciplexes are particularly suitable for use as labels in sequencing operations because it is possible readily to select the plurality of excimers or exciplexes required for the sequencing reaction such that they are readily individually detectable.

It is particularly preferred that exciplexes be used for the sequencing reaction because the emission characteristics thereof can be tuned within families of exciplex donar-acceptor pairs by varying the relative ionisation potential-electron affinity of the partners. For example, an exciplex formed from N, N-diethylamine with chrysene emits at ca 420nm but one formed from N, N-diethylamine with perylene emits at ca 520 nm. Fluorescence lifetimes can also be altered by this means so that a particularly suitable method of detection is by time resolved fluorescence. Because lifetimes can be resolved in principle better than the broad emission maxima of fluorescing exciplexes then at least four such "tags" could be envisaged to operate simultaneously in the same solution.

The invention may be applied to the sequencing of DNA, RNA and protein and peptide nucleic acids.

The method of the invention may be conducted in various ways.

In one preferred embodiment, the sequencing method is conducted in a manner analogous to the Sanger sequencing method utilising fluorescent labels but employing, as such labels, excimers or more preferably exciplexes. Thus, the invention the sequencing method may involve the use of chain terminating (i.e. "stop") nucleotides each labelled with detectably different excimers or exciplexes (or more correctly partners capable of forming such detectably different excimers or exciplexes). This preferred sequencing reaction may be conducted by employing the differently labelled stop nucleotides in a single reaction together with unlabelled nucleotides. At the end of the reaction, the different fragments produced may be separated on an electrophoresis gel and the various fragments resolved into bands

which may then be detected. Since the stop nucleotides are labelled with detectably distinguishable excimers or exciplexes it is possible to determine for each band the stop nucleotide with which it is terminated and thus to establish the sequence of the nucleic acid.

A further, but less preferred, possibly is to conduct four separate copying reactions each utilising "normal" nucleotides, a particular unlabelled "stop" nucleotide, and a primer labelled differently for each reaction with an excimer or exciplex, whereby a particular label is associated with the use of particular chain terminating nucleotide. The four separate reaction mixtures may then be combined and the fragments detected as previously.

A further possibility is as outlined in the previous paragraph save that for any one reaction a particular chain extending nucleotide is labelled with an excimer or exciplex whereby, as above, label is associated with the use of terminating a particular chain terminating nucleotide. Again, as previously, the four separate reaction mixtures may then be combined and the fragments detected for obtaining the sequence of the nucleic acid.

All embodiments of the invention require that a nucleotide be provided with the partners of an excimer or exciplex positioned in the correct relationship to each other for formulation on photo-irradiation of an excimer or exciplex. As indicated the partners may be provided on a chain terminating nucleotide, a chain extending nucleotide or on a nucleotide of a primer. Thus according to a further aspect of the invention there is provided a nucleotide or a nucleotide analogue labelled with excimer or exciplex forming partners. According to a still further aspect of the invention there is provided an oligonucleotide primer labelled with excimer or exciplex forming partners. The invention is thus able to provide, for example, a set of four (or more) "stop" nucleotides labelled with partners forming detectably different excimers or exciplexes. The invention is also able to provide a set of "normal", i.e. chain extending, nucleotides labelled with the partners of detectably different excimers or exciplexes. Similar considerations apply to the primers of the invention.

Excimers which may be employed in the invention are for example as disclosed in the review entitled "Photophysics of Aromatic Molecules" by J.B. Birks (Wiley – Interscience 1970). Particular (but non-limiting) examples include pryene (for which the excimer fluoresces at 470 nm as compared to 378 nm, 400 nm and 420 nm (shoulder) of the monomers, benzene, toluene, mesitylene, 1-methylnaphthalene, 2-methylnaphthalene, 1,6-dimethyl naphthalene, 1-fluoronaphthalene, acenaphthene, and 1,2-bezanthracene. A further possibility is anthraquinol (synthesis disclosed for example by A. Castellan, Tetrahedran Letter (1983) 24 5215) which emits at 580 nm.

Exciplexes for use in the invention may be as disclosed, for example, by "The Exciplex" (A. Weller; ed M. Gordon and W.R. Ware, Academic Press N.Y. 1975) and Z. Phys. Chem (1970) 69 1983. There are very many more specific examples in the literature and these include pyrene and an N, N-dialkyl amine, perylene and an N,N-dialkyl amine, a metalloporphyrin and a nitroaromatic compound (see J. Amer. Chem. Soc. (1971), 93, 7093; (1974), 96, (6349), and between a phthalocyanine and a nitroaromatic (see Inorg. Chem., (1983), 22, 1672).

Exciplexes have the advantage that it is possible by altering the electron affinity and ionisation potential of the contributing partners to "tune" the emission wavelength if the complex including the wavelength and temporal characteristics, as may be used in time-resolved fluorescence. For example, an exciplex formed from N,N-diethylamine with chrysene emits at ca 420 nm but one formed with N,N-diethylamine with perylene emits at ca 520 nm.

More particularly, the emission characteristics can be tuned in a predictable sense by the fine chemical structures of the partners, for example the emitted light frequency being linearly related to the difference in electron donor/electron acceptor strengths of the partners (see for example D.RehM, S. Naturforsch (1970) Vol 25a 1442-1447; J.B. Birks "Photophysics of Aromatic Molecules" published by Wiley Interscience, London.

It will generally be convenient for the excimer or exciplex partners to be connected by a linker which allows the partners to come into the correct relationship for excimer or exciplex formation on photo-irradiation, the linker itself being bonded to a nucleotide. Possible linkers are discussed below with specific reference to exciplexes but are also applicable to excimers.

In the case of an exciplex, it is particularly preferred that there are two exciplex forming partners each having at least one aromatic nucleus and are connected by a saturated aliphatic chain having a length and flexibility to allow the partners to come into exciplex forming relationship on irradiation with light of the appropriate wavelength. By the term "saturated aliphatic chain" we mean that "line" of atoms which links the two exciplex forming partners does not include an atom in that "line" which has more than one bond to another single atom. Thus, for example the carbon atom of a carbonyl group is regarded as being an unsaturated atom. The presence of unsaturated atoms in the aliphatic linker chain will restrict rotation (about the unsaturated bond) and act to prevent the (potential) exciplex partners coming into exciplex forming relationship.

It should be noted that the saturated aliphatic linker chain may itself be substituted (possibly with groups containing unsaturated atoms provided that such atoms are not in the "line" of atoms connecting the two exciplex forming partners).

Preferably the saturated aliphatic linker comprises 2 to 9 saturated atoms as the link between the two exciplex-forming partners. More preferably the linker comprises 2 to 4, and ideally 3, saturated atoms as the link. The saturated atoms of the linker may be carbon atoms (e.g. provided by methylene groups) or may be comprised partially or wholly of other atoms, e.g. nitrogen, sulfur or oxygen.

Preferred examples of linker include $-CH_2-CH_2-$, $CH_2-CH_2-CH_2$, and $-CH_2-NH-CH_2-$ in which the free terminal bonds are bonded to the exciplex forming partners.

The exciplex forming partners will comprise donor and acceptor moieties (each incorporating at least one aromatic nucleus) which may be selected (by way of example only) from residues of any of the following compounds, namely

(i) benzene, naphthalene, anthracene, phenanthrene, pyrene and chrysene, phenanthrene;

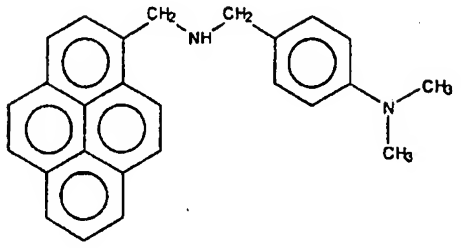
(ii) substituted derivatives of compounds identified under (i), particularly (but not exclusively) amino or substituted amino derivatives such as N-alkylamino substituted derivatives (where the alkyl groups preferably have 1 or 2 carbon atoms); and N,N-dialkyl substituted derivatives where the two alkyl groups may be but need not necessarily be identical to each other; and

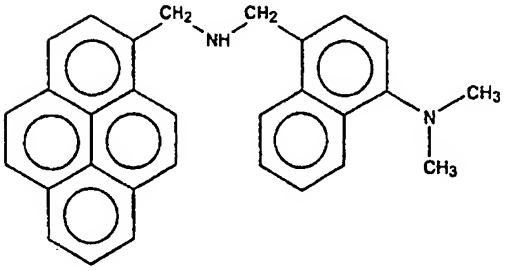
(iii) phthalimide or substituted derivatives thereof

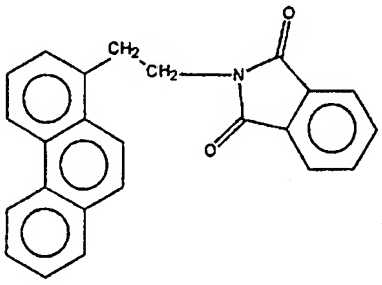
For the residues identified under (i) and (ii), the saturated aliphatic linker will preferably be bonded directly to the carbon atom of the aromatic ring. In the case of (iii) the linker will be bonded to the imide ring which (because the phthalimide residue is the exciplex partner) does not constitute part of the saturated aliphatic linker.

It is particularly preferred that the donor exciplex partner is a 1-pyrenyl group and the acceptor is an amino, N-alkylamino or N,N-dialkylamino substituted benzene or naphthalene nucleus. The alkyl groups are preferably methyl or ethyl groups. Preferably the amino (or alkyl substituted amino group) is of the 4-position relative to the position at which the linker is bonded to the benzene or naphthalene.

Examples of suitable exciplexes are based on compounds of the following formulae:







Such compounds may be covalently attached via the linker group to a nucleotide. Thus for example, analogues of the above compounds may be prepared in which a functional group (eg. an amino group) is bonded to the linker and the functional group is used to bond the exciplex forming partners to a nucleotide.

Sequencing reactions are generally conducted in aqueous media which depending on the particular exciplex partners employed may, because of the polarity of the media, preclude detectable exciplex formation without additional steps being taken to achieve such formation and/or enhance detection.

Enhancement of exciplex signal may be achieved by employing at least one of the steps, in either order, of (i) at least partial removal of the relatively polar medium in which the labelled fragments were generated, and (ii) addition of a less polar medium.

In accordance with this embodiment of the invention, the relatively polar medium is at least partially removed from the sample, e.g. by vacuum or other dehydration technique (e.g. freeze drying). Depending on factors such as the particular exciplex partners, this (at least) partial removal of the relatively polar medium may be sufficient to give the required exciplex emission (given that the oligonucleotides have hybridized to the target nucleic acid). It is however possible, in accordance with this embodiment, to add to the (at least partially) dehydrated sample a solvent or solvent mixture which is less polar than the original medium. Thus for example, is the case where the original medium was water, the relatively less polar solvent may comprise acetonitrite or even lower polarity solvent such as methylcyclohexane. Mixtures of such solvents may also be used.

In a variation of this embodiment it is possible to add to the medium in which the fragments are generated (without prior removal of the original bulk medium) a solvent or solvent mixture of lower polarity than the original bulk medium. Examples of such solvents and solvent mixtures are as described for the first embodiment.

Depending on factors such as the exciplex partners, the addition of the solvent or solvent mixture may be sufficient to provide the required exciplex emission.

It is possible to incorporate in the medium in which the detection is to be detected an additive which provides a localised region of increased hydrophobicity.

Examples of additives which may be included in accordance with this embodiment of the invention include compounds capable of acting as a host in a host-guest complex for which the guest is formed of the exciplex forming partners. The type of cavity that can be added as external agent to develop the exciplex fluorescent signal include cyclodextrins, cyclophanes, calixarenes, crown ethers, cryptands and

other well known host-guest systems or analogues or mixed structures capable of providing a hydrophobic cavity able to sequester wholly or partially the exciplex partners in an exciplex forming relationship.

Cyclodextrins are known from studies on intramolecular exciplex assemblies to enhance exciplex emission in aqueous solution (G .S. Cox et al. JACS (1984) v106, p422 et seq.) depending on whether the cyclodextrin is α, β or γ and on the pH of the solution. To use the cyclodextrin method the medium in which the exciplex signal is to be developed has added thereto α, β, γ, or other cyclodextrin, as is appropriate for the particular pair of exciplex partners (the particular cyclodextrin cavity size depends on the particular pair of partners being used).

Cyclophanes, calixarenes, crown ethers and many other examples from the families of host-guest chemistry can also serve as additives that provide a cavity in the manner of cyclodextrin. An example of such a cyclophane exciplex system is described in D.R.Benson & J. Fu, Tetrahed. Lett. (1996) v37 pp4833-4836. To use the cyclophane method, the binary split-probe system consisting of the target strand and the 2 shorter complementary oligos chemically modified with the exciplex partners has added to it a cyclophane appropriate for the particular pair of exciplex partners (the particular cyclophane cavity size being constructed for the particular pair of partners being used).

Alternatively, the additive may for example be a surface active agent which in the bulk phase forms aggregates (e.g. micelles) within which exciplex may form. The surface active agent is preferably a cationic surface active agent, most preferably a quaternary ammonium salt having at least one chain of 4 or more carbon atoms bonded to the quaternary nitrogen atom. Preferably this carbon chain has at least 8 and even more preferably at least 10 carbon atoms. Preferably also the quaternary ammonium salt has at least two, and preferably three, C_{1-3} alkyl groups, particularly methyl groups. The preferred quaternary ammonium cations include the hexadecyltrimethyl ammonium, cetyldimethylethyl ammonium, tetradecyltrimethyl

ammonium, decyltrimethyl ammonium and dodecyltrimethyl ammonium ions. The anion is preferably bromide. The concentration of the surface active agent is preferably 1×10^{-5} to 1×10^{-6} M although we do not preclude amounts outside this range provided that the amount used is not so high as to precipitate the nucleic acid.

In accordance with a further embodiment of the invention there is included in the medium in which the detection is being affected at least one compound capable of developing an exciplex fluorescence signal.

Examples of additives which may be included in accordance with this embodiment of the invention include compounds capable of acting as a host in a host-guest complex for which the guest is formed of the first and second moieties in exciplex relationship. The host compound may be one having a cavity in which the guest is received. The type of cavity that can added as external agent to develop the exciplex fluorscent signal include cyclodextrins, cyclophanes, calixarenes, crown ethers, cryptands and other well known host-guest systems. Examples of hosts as described more fully for the previous aspect of the present invention are also applicable to this embodiment. However in accordance with this embodiment it is not essential that the host provides a localised hydrophobic environment for the excimer or exciplex partners.

It is known that exciplexes in water can stabilised by polyanions. Consequently a further possibility for this embodiment is for the additive to be a polyanion.

The polyanion may, for example be provided by a polysulfate such as poly(vinyl sulfate), chondroitin sulfate A, B or C, heparin, a polyphosphate, a nucleic acid or semi-synthetic nucleic acid (including PNA's and peptide nucleic acids), a polycarboxylate, or a polyanionic polymer containing at least two of carboxylate, phosphate and sulfate groups.

In accordance with a further embodiment of the invention it is possible to use a magnetic field to enhance exciplex emission. This allows exciplex emission to be produced even in polar solvents. The magnetic field may for example be up to 5T, more preferably up to 2T, e.g. up to 1T.

The increase in exciplex luminescence with the magnetic field will depend on factors such as (a) the particular exciplex partners, (b) the particular solvent (or solvent mixture), (c) the magnetic field strength, and the ionic strength of the bath media.

Detection of the exciplex may be by means of wavelength detection but is more preferably by the use of time resolved fluorescence or by a combination of these approaches. Exciplexes may also be detectable by other spectroscopic methods including circular dichroic spectral effects as has been described for excimer systems (see for example H. Mihara, Y. Tanaka, T Fujimoto, and N. Nishino J. Chem. Soc. Perkin Trans 2 (1995) p1133-1140.

It is possible for the analysis to be such that more than one type of exciplex may be formed whereby the various types of exciplex are separately detectable to identify particular aspects of the nucleic acid under investigation. Thus for example the method may involve formation of two or more exciplexes which are detectable at different wavelengths. More preferably however detection of the different exciplexes will be by means of time resolved fluorescence or by a combination of wavelength dependent and time resolved methods.

The method of the present invention may involve an analysis for determining a characteristic lifetime of an exciplex using the technique described more fully in our co-pending application PCT/GB99/02047 entitled "Sample Analysis", the disclosure of which is incorporated herein by reference. More particularly, the technique of the co-pending application is a method of analysis for determining a characteristic lifetime of a sample, the method comprising exciting active elements in the sample either continuously or using pulses having an inter-pulse separation which is of the

order of or less than the characteristic lifetime of the active elements, detecting quanta emitted by the active elements in the sample to obtain a detected signal, correlating the detected signal with itself and analysing the correlated signal to derive the characteristic lifetime, wherein the number of active elements in the sample and the intensity of the excitation are such that quanta are detected in a stream in which individual quanta are distinguishable from each other. As applied to the present invention, the sample is an excimer or exciplex.

The technique of the present invention may be effected using exciplexes whereof the periodicity has been altered using the techniques disclosed in ourabovementioned co-pending PCT/GB99/02047, the disclosure of which is incorporated herein by reference. The co-pending application covers a method of altering the periodicity of a dyestuff by altering the lifetime of at least one lower energy state of the dyestuff by the alteration of the dyestuff's environment by means which include chemical and/or physical means. As applied to the present invention, the dyestuff is an excimer or exciplex.

The invention is illustrated by the following non-limiting Examples for which results are shown in the accompanying drawings, in which:

Figure 1 shows exciplex emission of compound **3** in pH 9 tris buffer (excitation was at 350 nm);

Figure 2 shows fluorescence emission of **3-pTGTTTGGC** (10^{-4} M) in pH 7 and pH 10 0.01M Tris buffer;

Figure 3 shows fluorescence emission of **3-pTGTTTGGC** (10^{-4} M) in 0.01M Tris buffer pH 8.5 in the presence of 50% v/v acetonitrile, methanol and tetrahydrofuran (THF) as well as in pure THF;

Figure 4 shows fluorescence emission of **dCGATTCTGp-3** in 0.01M Tris buffer pH 8.8, in amedium prepared from a 10%(v/v) of Tris pH 8.8 plus 90% of tetrahydrofuran (THF), as well as in pure THF; and

Figure 5 shows fluorescence emission of **dCGATTCTGp-3** at various concentrations of THF.

Example 1

This Example illustrates the labelling of oligonucleotide primer with exciplex forming partners and also the detection of exciplex formation on irradiation of the primer. As such, the Example illustrates the feasibility of using exciplex labelled primers, and thus also exciplex labelled nucleotides in sequencing reactions.

The 8-mer oligodeoxyribonucleotide pTGTTTGGC was condensed through its 5'-phosphate site with N-(2-aminoethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine (**3**) to produce the labelled oligonucleotide designated herein as **3-pTGTTTGGC**. The synthetic procedures used were as described in Appendix A.

The exciplex emission spectrum of **3** in pH 9 tris buffer is illustrated in Fig 1. For **3-pTGTTTGGC** a long tail of emission was seen in the region of 480 nm, with only a weak peak in this region at pH 7 or 10 (**Figure 2**). The effects of added organic solvent on the exciplex emission of **3-pTGTTTGGC** (**Figure 3**) were tested: the addition of (i) acetonitrile or (ii) methanol (to 50% v/v) to **3-pTGTTTGGC** in pH 8.5 Tris buffer gave no significant increase in emission at 480 nm, although there was some change detected for (iii) tetrahydrofuran. The spectrum of **3-pTGTTTGGC** in (iv) pure tetrahydrofuran (**Figure 3**) shows strong emission at 485 nm, typical of the exciplex.

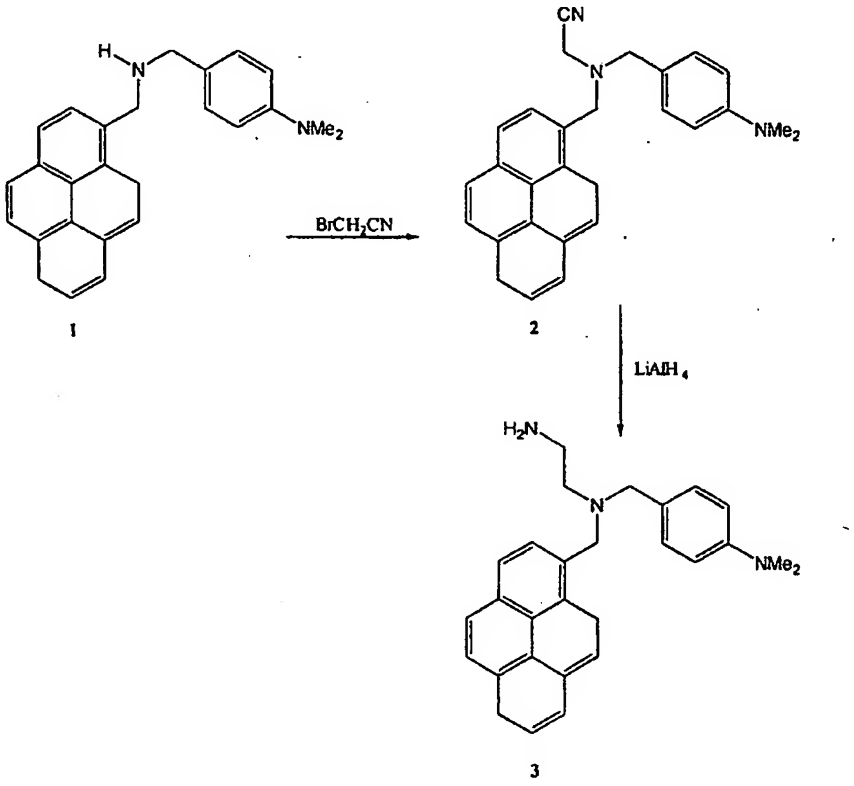
Example 2

The 8-mer dCGATTCTGp was labelled on the 3' phosphate with compound **3** to produce the labelled oligonucleotide designated herein as **dCGATTCTGp-3**. The emission spectra of **dCGATTCTGp-3** are shown in **Figure 4**: the exciplex emission band around 485 nm is clearly seen in (v) pure tetrahydrofuran and in (vi) a medium prepared from 0.01M Tris buffer at pH 8.8 (10% v/v) and tetrahydrofuran (90% v/v). The exciplex emission spectrum in 0.01m Tris buffer at pH 8.8 is shown in Fig 4 as curve (vii). With reference to Fig 5, the exciplex emission band at 480 nm is seen to increase in distinctness on going from (viii) 50, to (ix) 75 to (x) 92.5 % THF in the Tris buffer pH8.8 (in these experiments the solution was diluted by addition of THF and so the concentration of fluorophore also decreased.

APPENDIX A

Synthesis of N-(2-aminoethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine (3)

This was carried out according to Scheme 1



1 2 3

Synthesis of N-(cyanomethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine, **2**

Bromoacetonitrile (0.62mg, 0.52 mmol 0.035 ml) was added drop-wise to a stirred solution of N-(4-dimethylaminobenzyl)-N-1-pyrenyl)amine (0.19g, 0.52 mmol) and potassium carbonate (0.071g, 0.52 mmol) in acetone (0.5 ml) at 0°C. The reaction mixture was stirred at room temperature for 3h and then the solvent removed *in vacuo*. The resultant solid was triturated with water (15 ml) and extracted with diethyl ether (2 x 20 ml). The combined organic extracts were washed with water (2 x 10 ml), dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to give N-(cyanomethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine (2) as a yellow solid,

which after chromatography on silica gel with dichloromethane and hexane as solvent (9:1) was isolated as a white product (0.08g, 38%).

Synthesis of N-(2-aminoethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine, **3**

To a solution of N-(cyanomethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine (**2**, 0.06g, 0.15 mmol) in dry tetrahydrofuran (1 ml) was added $LiAlH_4$ (0.05g, 1.3mmol) and the mixture refluxed for 5 h after which the complex was decomposed by adding aqueous potassium hydroxide (2 ml, 40%). This mixture was extracted with diethyl ether (2 x 15 ml) after addition of a further aliquot of water (1 ml) and the combined ethereal extracts dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to give an oily yellow residue. Chromatography on Varian Mega BondElut SI(2g silica gel) with dichloromethane and a few drops of aq. NH_4OH yielded **3** as a yellow, oily product (0.05g, 81%).

DNA Labelling

To give **3-pTGTTTGGC**, the 8-mer oligodeoxyribonucleotide pTGTTTGGC was condensed through its 5'-phosphate site with N-(2-aminoethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine (**3**) as follows. **3-pTGTTTGGC** was synthesised by a two-step procedure. First, the cetyltrimethylammonium salt of 5'pTGTTTGGC was obtained by stepwise addition of 8% aqueous cetyltrimethylammonium bromide (100µl, 20µlx5) to a solution of the lithium salt of the oligonucleotide (1µmol) in 0.3 ml of water, with centrifugation on each addition, until no more precipitation was observed. The supernatant was removed, the precipitate dried *in vacuo* overnight over P_2O_5, and the cetyltrimethylammonium salt of the oligonucleotide (1µmol) dissolved in 0.4 ml of DMF. Triphenylphosphine (13.2 mg, 50µmol) and 2',2'-dipyridyl disulfide (11.2 mg, 50 µmol) were added, and after 10 min 4-*N',N'*-dimethylaminopyridine (6.2 mg, 50µmol) was added. After 15 minutes incubation at 20°C, N-(2-aminoethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine (**3**) (5.4mg, 20µmol) and triethylamine (28µl, 20µmol) was added and

the reaction mixture incubated at 25°C for 1h, followed by precipitation by 25 ml of acetone containing 2% $LiClO_4$. The oligonucleotide conjugate (**3-pTGTTTGGC**) was separated from unreacted precursor (**3**) by reverse-phase HPLC (0 to 40% acetonitrile gradient) to give total yield of product of 90% based on starting oligonucleotide. The incorporation of the structure from 3 into the oligonucleotide was confirmed by its HPLC behaviour, and by UV-visible specrophotometry, giving a charactersitic absorbance at 350 nm maximally for the chromophore corresponding to **3**.

The synthesis of **DCGATTCTGp-3** was as described above but using dCGATTCTGp labelled on the 3' phosphate with compound **3.**

Claims

1. A method of determining the sequence of a nucleic acid involving the use of a label wherein the label is an excimer or exciplex.

2. A method of sequencing a nucleic acid by generating labelled nucleic acid fragments derived from the nucleic acid and detecting fragments to determine the sequence of the nucleic acid wherein the label of the fragments is an excimer or an exciplex.

3. A method as claimed in claim 1 or 2 which comprises effecting a single copying reaction of the nucleic acid to be sequenced using chain extending nucleotides and an appropriate number of chain terminating nucleotides each labelled with the partners of a detectably different excimer or exciplex, the method further comprising separating the labelled fragments produced and detecting the labels to determine the sequence of the nucleic acid.

4. A method as claimed in any one of claims 1 to 3 wherein the label is an exciplex.

5. A method as claimed in claim 4 wherein the two exciplex forming partners each have at least one aromatic nucleus and are connected by a saturated aliphatic chain having a length and flexibility to allow the partners to come into exciplex forming relationship on irradiation with light of the appropriate wavelength.

6. A method as claimed in claim 5 wherein the saturated aliphatic linker comprises 2 to 9 saturated atoms as the link between the two exciplex-forming partners.

7. A method as claimed in claim 6 wherein the linker comprises 2 to 4 saturated atoms as the link.

8. A method as claimed in claim 3 wherein the linker comprises 3 saturated atoms as the link.

9. A method as claimed in claim 8 wherein the linker is selected from $-CH_2-CH_2-$, $CH_2-CH_2-CH_2$, and $-CH_2-NH-CH_2-$ in which the free terminal bonds are bonded to the exciplex forming partners.

10. A method as claimed in any one of claims 4 to 9 wherein the exciplex forming partners are selected from

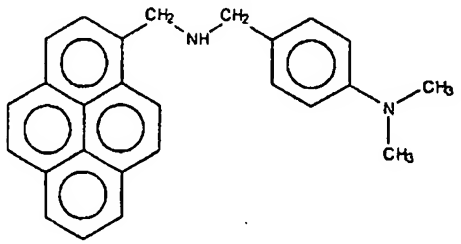
(i) benzene, naphthalene, anthracene, phenanthrene, pyrene and chrysene, phenanthrene;

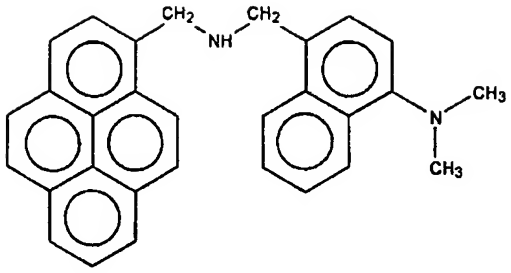
(ii) substituted derivatives of compounds identified under (i); and

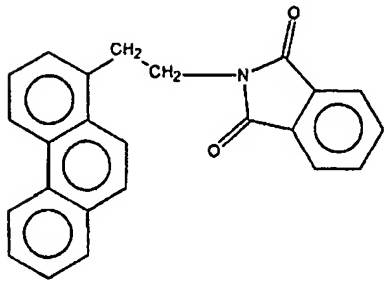
(iii) phthalimide or substituted derivatives thereof.

11. A method as claimed in claim 10 wherein the donor exciplex partner is a 1-pyrenyl group and the acceptor is an amino, N-alkylamino or N,N-dialkylamino substituted benzene or naphthalene nucleus.

12. A method as claimed in claim 11 wherein the exciplex label is based on one of the following compounds







13. A method as claimed in any one of claims 4 to 12 wherein the sequencing reaction is conducted in an aqueous medium.

14. A method as claimed in claim 13 wherein an additive is incorporated in the medium to provide a localised region of increased hydrophobicity for exciplex formation.

15. A method as claimed in claim 14 wherein the additive is a compound capable of acting as a host in a host-guest complex for which the guest is formed of the exciplex forming partners.

16. A method as claimed in claim 15 wherein the host is a cyclodextrin, cyclophane, calixarene, crown ether, cryptand or analogous structure.

17. A method as claimed in claim 14 wherein the additive is a surface active agent.

18. A method as claimed in claim 17 wherein the surface active agent is quaternary ammonium salt having at least one chain of 4 or more carbon atoms bonded to the quaternary nitrogen atom.

19. A method as claimed in any one of claims 4 to 13 wherein exciplex formation is stabilised by a polyanion.

20. A method as claimed in claim 19 wherein the example is provided by a polysulfate such as polyvinyl sulfate, chondroitin sulfate A, B or C, heparin, a polyphosphate, a nucleic acid or semi-synthetic nucleic acid, a polycarboxylate or a polyanionic polymer containing at least two of carboxylate, phosphate and sulfate groups.

21. A method as claimed in any one of claims 4 to 13 wherein an external magnetic field is applied to enhance the exciplex signal.

22. A method as claimed in any one of claims 4 to 13 wherein enhancement of exciplex signal is achieved by employing at least one of the steps, in either order of (i) at least partial removal of the relatively polar medium in which the labelled fragments were generated, and (ii) addition of a less polar medium.

23. A method as claimed in any one claims 1 to 22 wherein the nucleic acid is DNA, RNA or a protein or peptide nucleic acid.

24. A method as claimed in any one of claims 1 to 23 wherein the excimer or exciplex is detected by time resolved fluorescence.

25. A method as claimed in any one of claims 1 to 23 wherein the excimer or exciplex is detected by circular dichroism.

26. A nucleotide or nucleotide analogue labelled with the partners of an excimer or an exciplex.

27. A nucleotide analogue as claimed in claim 26 which is a "stop" nucleotide, e.g. a dideoxy nucleotide.

28. An oligonucleotide primer labelled with the partners of an excimer or an exciplex.

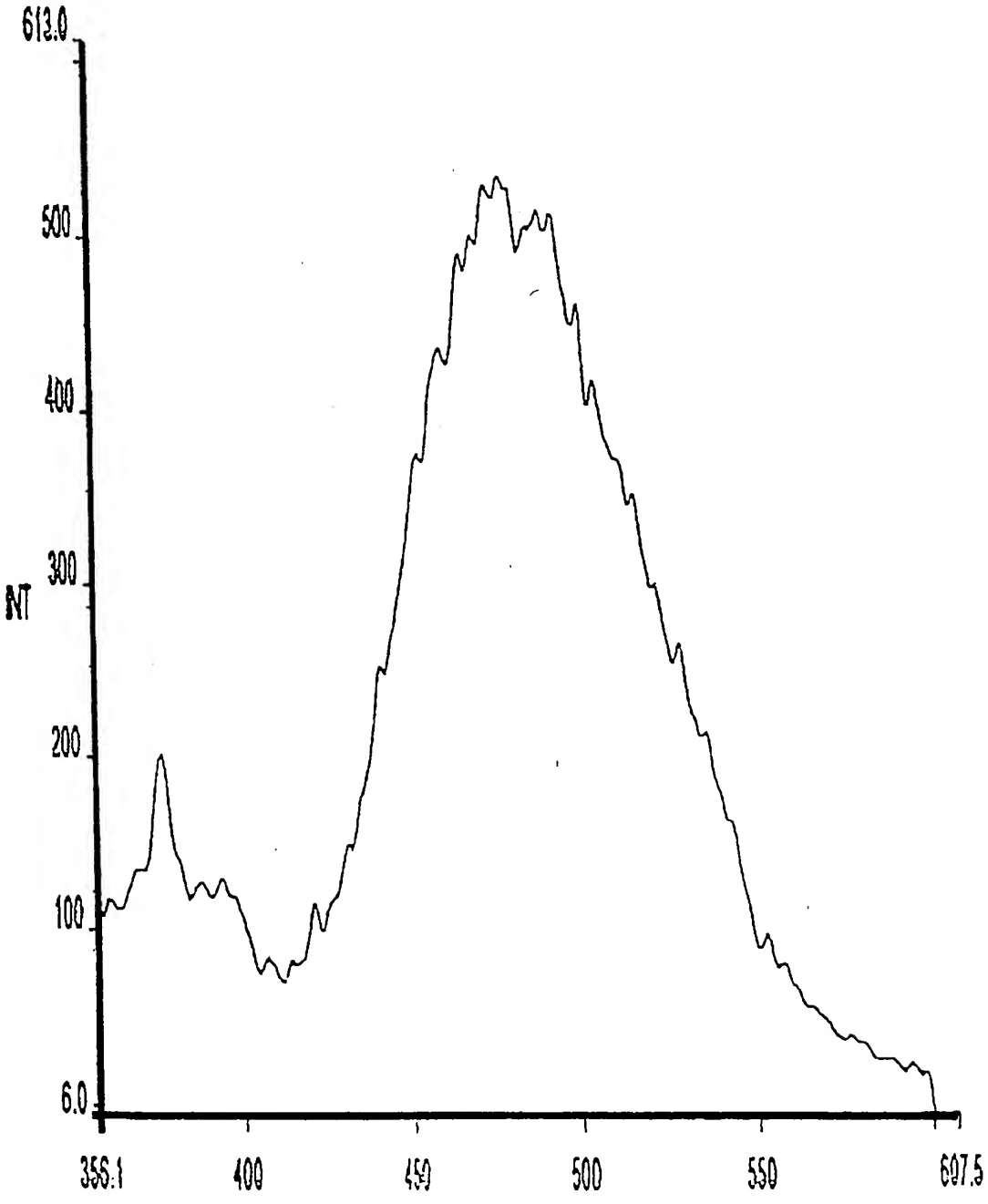


FIG. 1

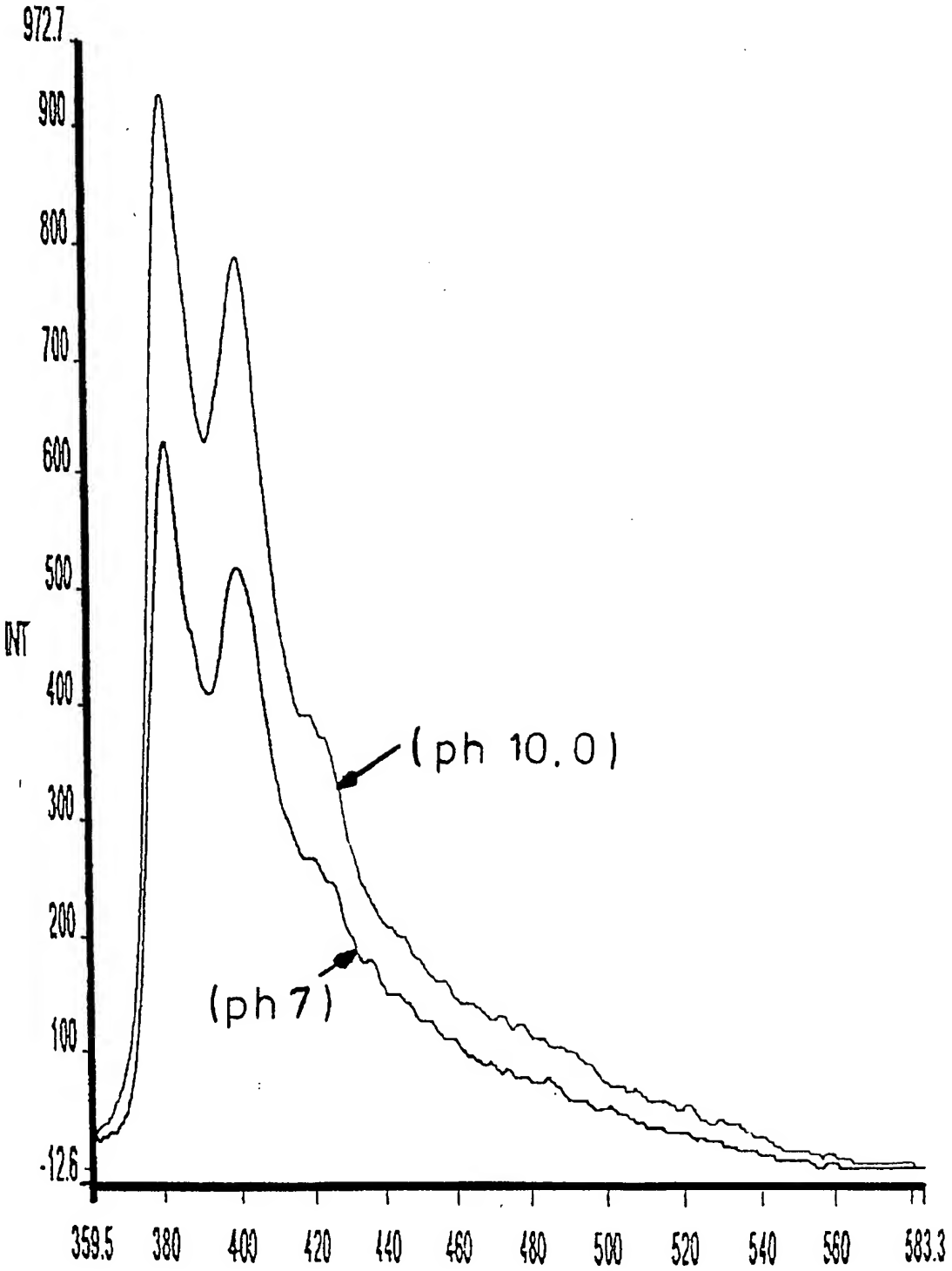

972.7
900
800
700
600
500
INT
400
300
200
100
-12.6
(ph 10.0)
(ph 7)
359.5
380
400
420
440
460
480
500
520
540
560
583.3
nm

FIG. 2

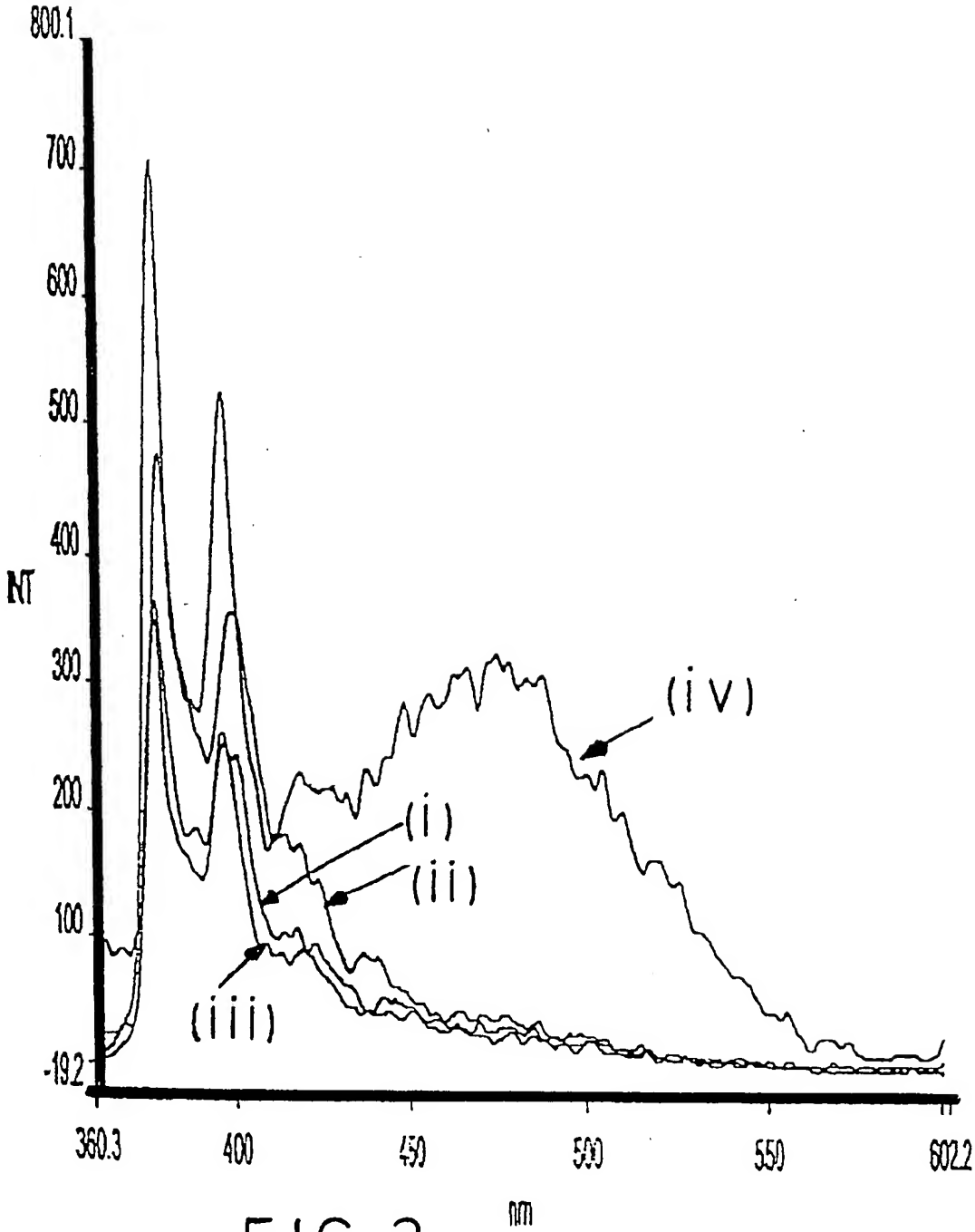

800.1
700
600
500
400
300
200
100
-19.2
INT
360.3
400
450
500
550
602.2
nm
(i)
(ii)
(iii)
(iv)

FIG. 3

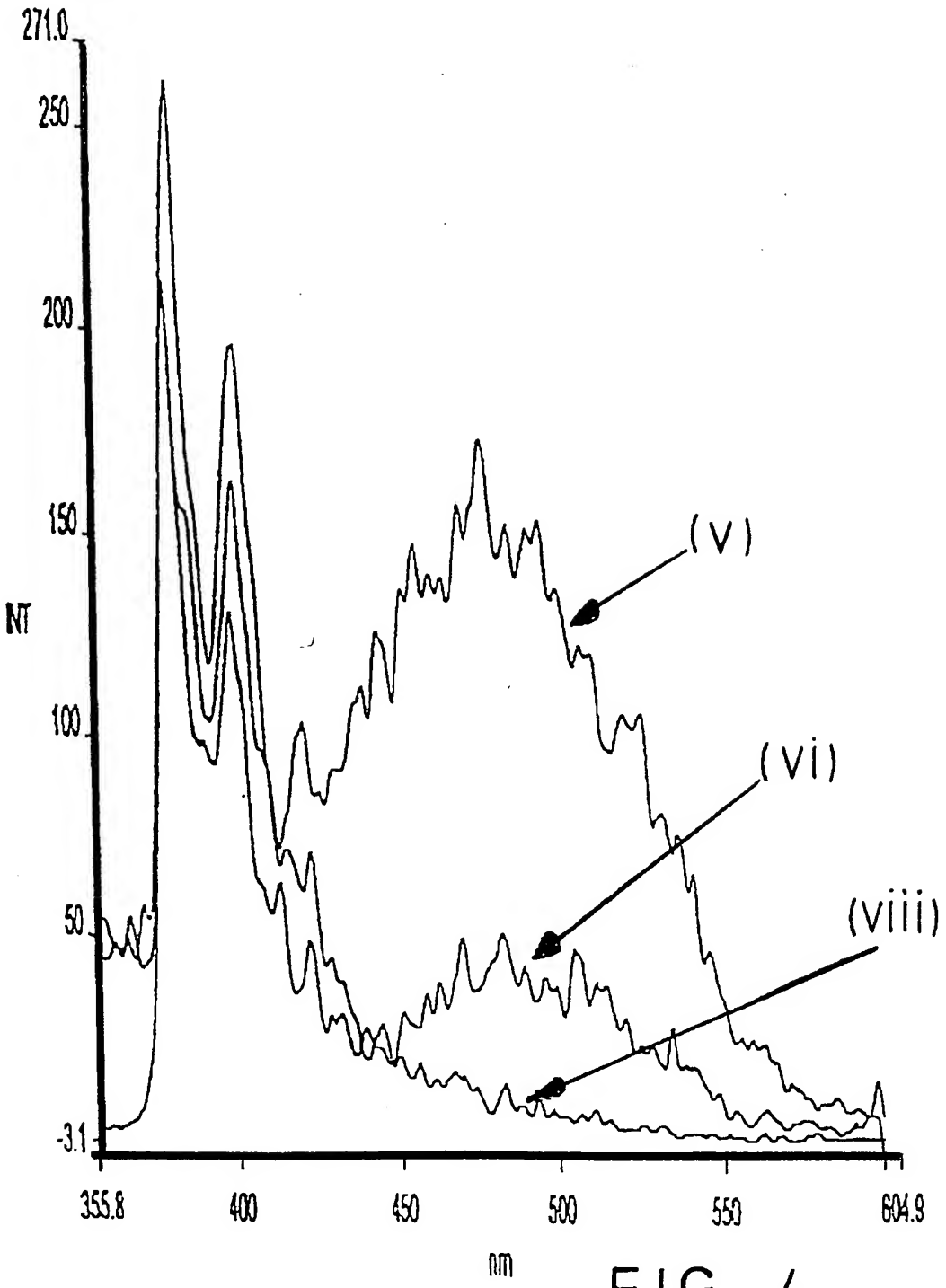

271.0
250
200
150
100
50
-3.1
INT
(v)
(vi)
(viii)
355.8
400
450
500
550
604.9
nm

FIG. 4

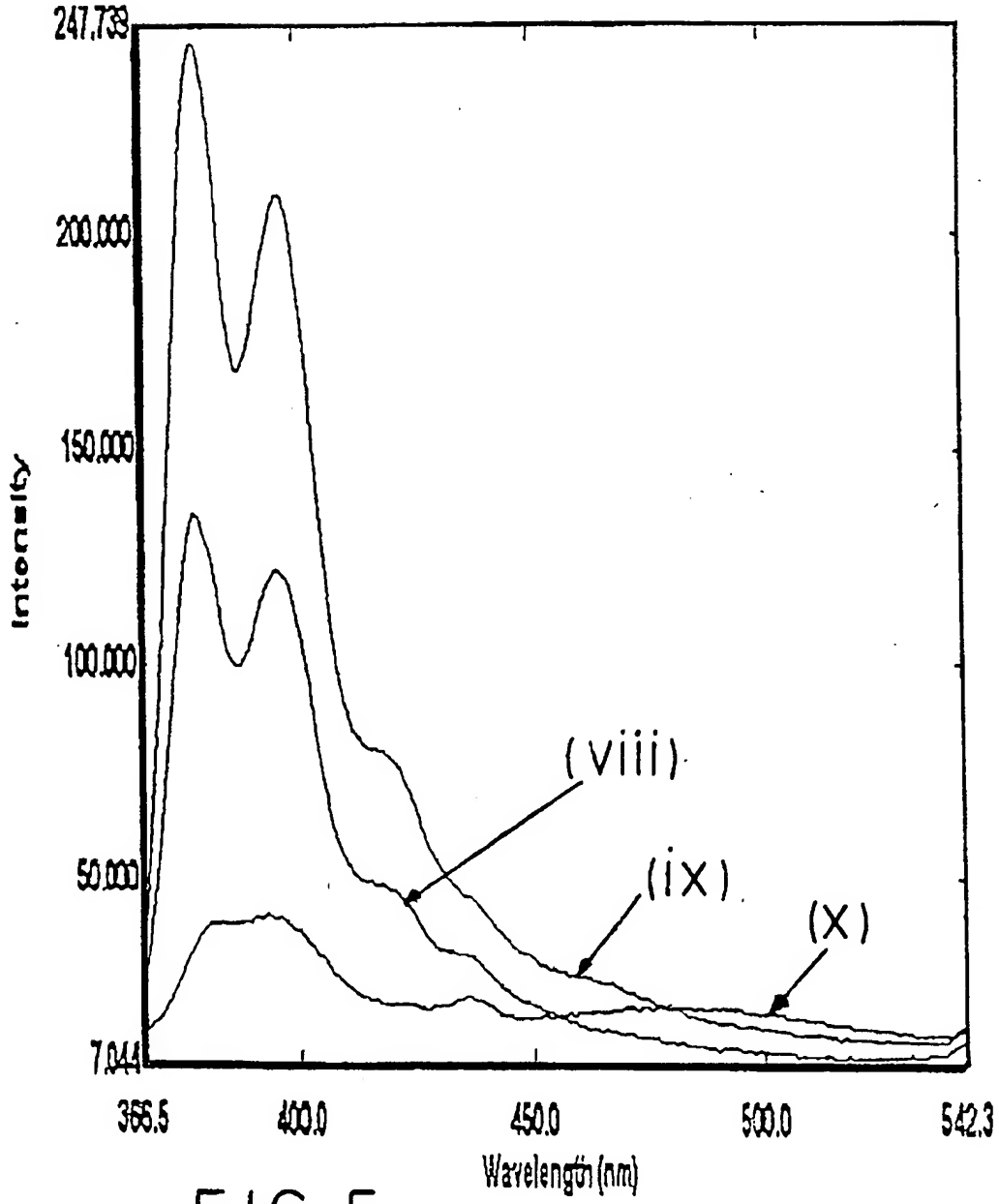

247.739
200.000
150.000
100.000
50.000
7.044
Intensity
(viii)
(ix)
(x)
366.5
400.0
450.0
500.0
542.3
Wavelength (nm)

FIG. 5

INTERNATIONAL SEARCH REPORT

Int. .tional Application No
PCT/GB 99/04208

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C07H19/04 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 31834 A (INCYTE PHARMA INC) 23 July 1998 (1998-07-23) the whole document ---	1-28
X	WO 98 14612 A (UNIV CALIFORNIA) 9 April 1998 (1998-04-09) the whole document ---	1-28
X	WO 95 21266 A (UNIV CALIFORNIA) 10 August 1995 (1995-08-10) page X ---	1-28
X	US 5 332 659 A (KIDWELL DAVID A) 26 July 1994 (1994-07-26) the whole document ---	1-4, 13-28
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 April 2000	09/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Molina Galan, E

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. .tional Application No
PCT/GB 99/04208

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 25518 A (SOCIETY FOR TECHNO INNOVATION ;MASUKO MASAYUKI (JP); EBATA KATSUYO) 22 August 1996 (1996-08-22) the whole document	1-4, 13-28
X	LIVAK K J ET AL: "OLIGONUCLEOTIDES WITH FLUORESCENT DYES AT OPPOSITE ENDS PROVIDE A QUENCHED PROBE SYSTEM USEFUL FOR DETECTING PCR PRODUCT AND NUCLEIC ACID HYBRIDIZATION" GENOME RESEARCH,US,COLD SPRING HARBOR LABORATORY PRESS, vol. 4, no. 6, 1 June 1995 (1995-06-01), pages 357-362, XP000522969 ISSN: 1088-9051 the whole document	26-28
A	SANGER F ET AL: "DNA SEQUENCING WITH CHAIN-TERMINATING INHIBITORS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA,US,NEW YORK, NY, vol. 74, no. 12, 1 December 1977 (1977-12-01), pages 5463-5467, XP000603873	
P,X	WO 99 28501 A (LEE MARTIN ALAN ;SECR DEFENCE (GB)) 10 June 1999 (1999-06-10) the whole document	26-28

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int .tional Application No
PCT/GB 99/04208

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9831834 A	23-07-1998	US 5804386 A	08-09-1998
		AU 5602298 A	07-08-1998
		EP 0943019 A	22-09-1999
		US 5952180 A	14-09-1999
		US 5814454 A	29-09-1998
WO 9814612 A	09-04-1998	US 5853992 A	29-12-1998
		AU 717018 B	16-03-2000
		AU 4742397 A	24-04-1998
		EP 0935670 A	18-08-1999
		GB 2317951 A,B	08-04-1998
WO 9521266 A	10-08-1995	US 5654419 A	05-08-1997
		AU 692230 B	04-06-1998
		AU 1736795 A	21-08-1995
		CA 2182516 A	10-08-1995
		DE 19581489 T	02-01-1997
		DE 29521620 U	13-11-1997
		EP 0743987 A	27-11-1996
		JP 9508525 T	02-09-1997
		US 5707804 A	13-01-1998
		US 5688648 A	18-11-1997
		US 6028190 A	22-02-2000
		US 5869255 A	09-02-1999
US 5332659 A	26-07-1994	US 5314802 A	24-05-1994
		US 5466578 A	14-11-1995
WO 9625518 A	22-08-1996	AU 694313 B	16-07-1998
		AU 4633896 A	04-09-1996
		CA 2213240 A	22-08-1996
		EP 0810291 A	03-12-1997
WO 9928501 A	10-06-1999	AU 1253399 A	16-06-1999
		GB 2333596 A	28-07-1999